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High Resolution Scanning Electron Microscopy of Fern Gametophytes: Applications of a Non-destructive Method

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Micromorphological data are increasingly used in taxonomic and developmental studies of pteridophytes (e.g. Zimmer, 1989). Although gametophytic data are considerably scarcer in the literature than sporophytic data, it is clear that gametophytes have much to offer, and scanning electron micrographs of gametophytic features are becoming increasingly common. The majority of published micrographs show fixed, dehydrated material illustrating structural or developmental points (e.g. Elmore & Adams, 1976; von Aderkas & Cutter, 1983; Whittier & Peterson, 1984a&b; Nester, 1985). A smaller number have included similarly processed material to illustrate taxonomic features (e.g. Tigerschiöld, 1985, 1989). A few studies have employed low-temperature (or cryo-) scanning electron microscopy (LTSEM) (e.g. Sheffield & Cutter, 1985; Sheffield & Farrar, 1988; Douglas & Sheffield, 1990) and one study included quantitative and qualitative comparisons of gametophytic material processed by conventional and low temperature methods (Attree & Sheffield, 1984). While it is clear that LTSEM offers superior preservation of delicate specimens, there are disadvantages with all existing SEM techniques. Low temperature work requires specialized, expensive equipment, and although free from the shrinkage and distortion seen in conventionally-prepared gametophytes, LTSEM specimens are not artefact-free (e.g. Jeffree et al., 1987; Moss, Howard & Sheffield, 1989). All the methods employed for gametophyte material to date are also destructive; the tissue cannot be used for any other purpose subsequent to micrograph production.

An investigation is in progress in this laboratory involving the cultivation of soil samples containing natural fern spore banks. A desire to obtain micromorphological and electrophoretic information from single gametophytes in such cultures prompted the present investigation. Our aim was to find a non-destructive method of obtaining good quality scanning electron micrographs, based on modifications by Jernstedt et al. (1991) of the method of Williams, Vesk & Mullins (1987) and Williams & Green (1988).

Materials and Methods

Gametophytes from artificial media and soil cultures were gently (but rapidly, to avoid dehydration) laid on the surface of freshly mixed Provil dental impression material (a two-component low viscosity type 1 silicone, ADA Nr. 19, manufactured by Bayer Dental Ltd., D5090 Leverkusen). After 3–5 minutes,

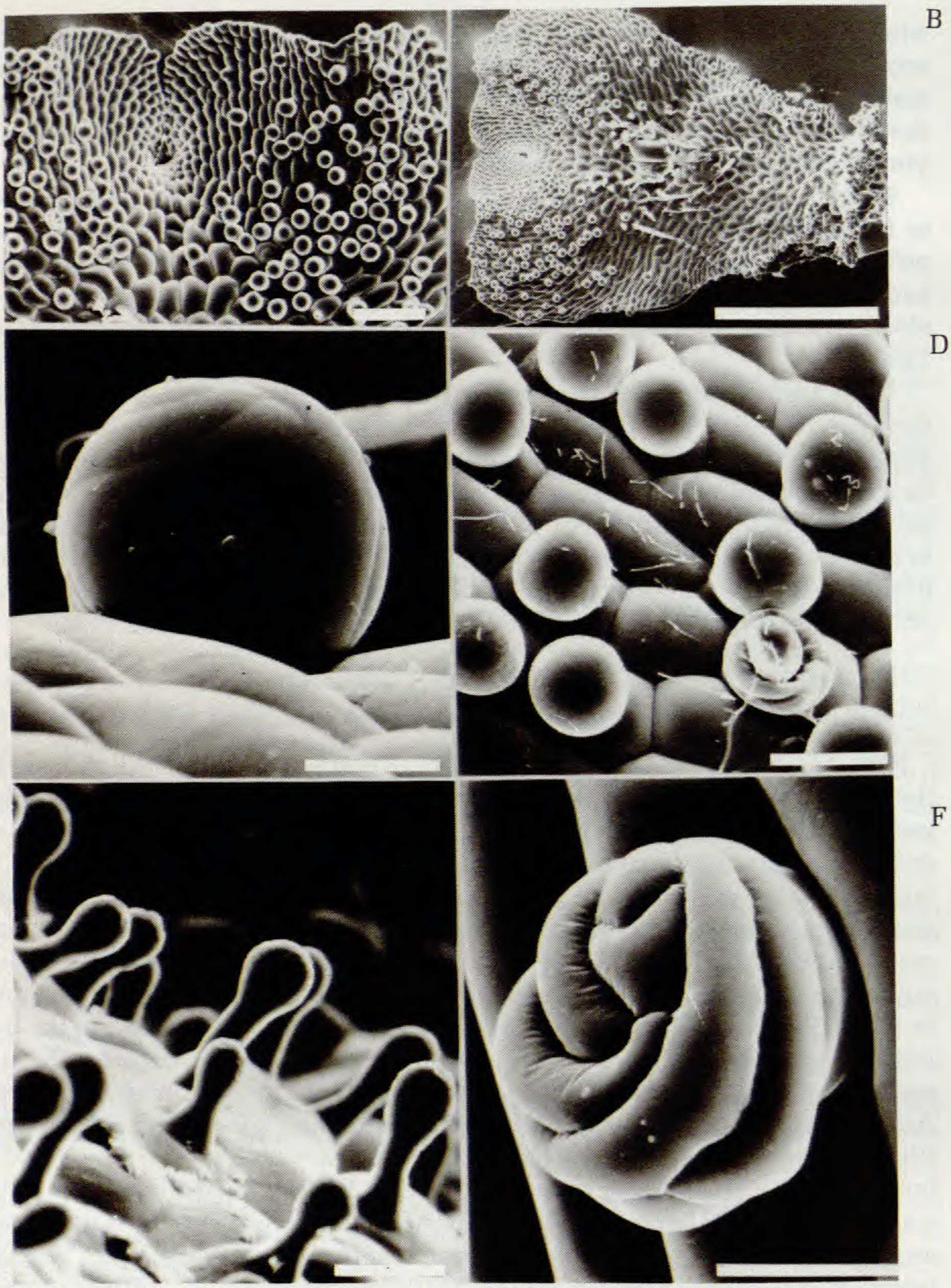
when the impression material had set, each gametophyte was removed. To accomplish this with the minimum of damage to the tissue, the impression material (which is flexible when set) surrounding the specimen was pushed downwards, causing the separation of the gametophyte and impression, and the gametophyte lifted out with a small blunt-ended instrument.

Gametophytes were then either (1) used directly for isozyme electrophoresis or (2) bisected; one half replaced on the growth medium and the other half processed for electrophoresis. Cultures were re-examined after several weeks and regeneration monitored and photographed. Horizontal starch gel electrophoresis followed established methods (e.g. Sheffield, Wolf & Haufler, 1989).

Casts were made from the impressions using freshly mixed Master Mend epoxy (a two part glue manufactured by Loctite). Air bubbles and inadequate penetration of the glue into small recesses were minimized by warming the glue on a foil-covered microscope slide on a hot plate, and administration of the mix using a pulled-out and rounded-off Pasteur pipette. The casts were left for 1h in a 60° C oven (this helps to expel air bubbles, cf. Jernstedt et al., 1991) removed from the impression, mounted on SEM stubs, coated with gold, examined and photographed in a Cambridge S90, S200 or 360 microscope.

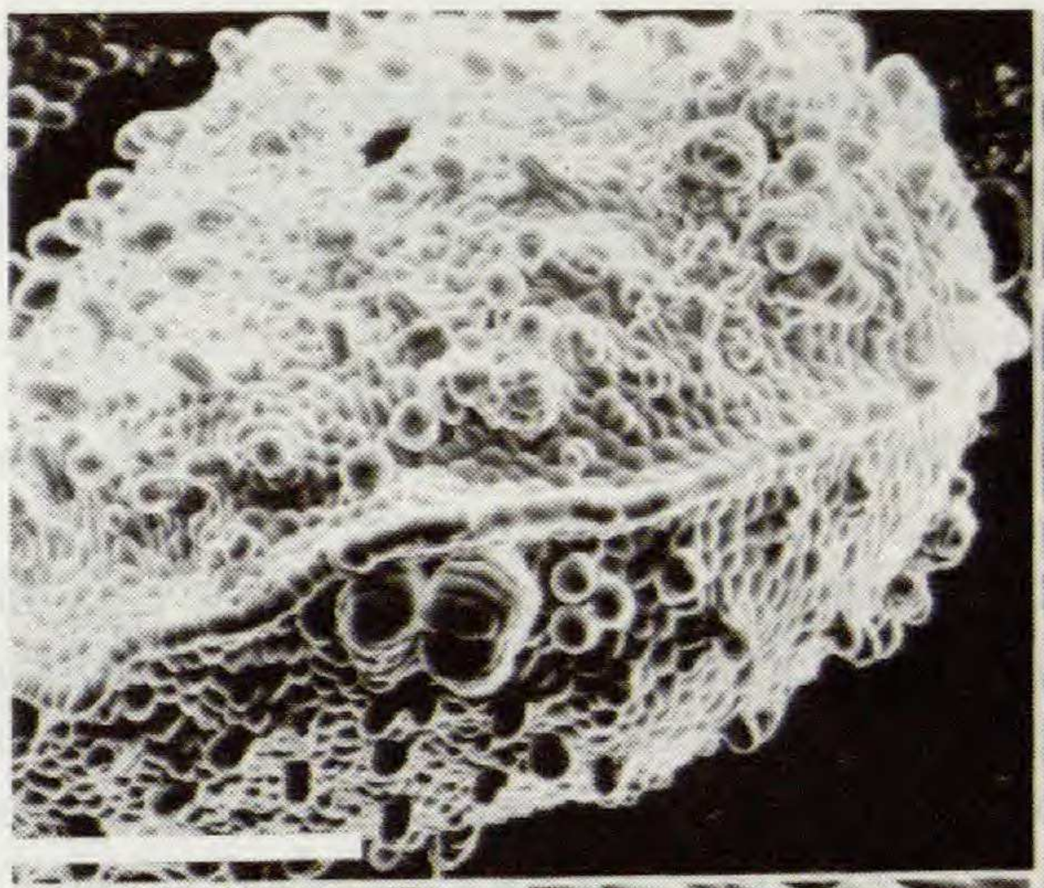
Results

Figs. 1A–F, 2A–D illustrate the quality of electron micrographs provided by the technique described, and micromorphological features of gametophyte casts. Whole gametophytes (1A), intact (1B) and senescent (1C) antheridia, and even superficial bacterial and fungal material (1C) were well represented. The technique also revealed fine details such as the dehydration and consequent puckering of antheridial jacket cells that accompanies maturation of *Osmunda regalis* antheridia (1D–1E) (this cannot be artefactual as fully turgid cells or younger antheridia were always found adjacent to stages such as that illustrated in 1E). Trichomes (1F) and larger three dimensional structures, such as the thick lobes of gametophytes grown on medium containing 5% sucrose (2A) were easily removed from the impressions and casts, and their form well preserved. Archegonial features were similarly well maintained (2B–D); 2C is included to illustrate the only minor problem caused by the technique, that of small air bubbles in the glue. When preparing the cast for Fig. 2C inadequate attention was given to eliminating air bubbles with the Pasteur pipette end, as the missing archegonial tips testify. Subsequent casts made more carefully from the same impression bore the entire structures. This demonstrates that multiple casts can be prepared from one impression, and that bubbles in the impression material seldom cause problems. Fig. 2E shows the phosphoglucoisomerase banding patterns of gametophytes from which impressions had been made (clear patterns were also obtained for the other isozymes tested: malate dehydrogenase, isocitrate dehydrogenase, shikimic dehydrogenase, and phosphoglucomutase). Fig. 2F shows the growth regenerated from half a

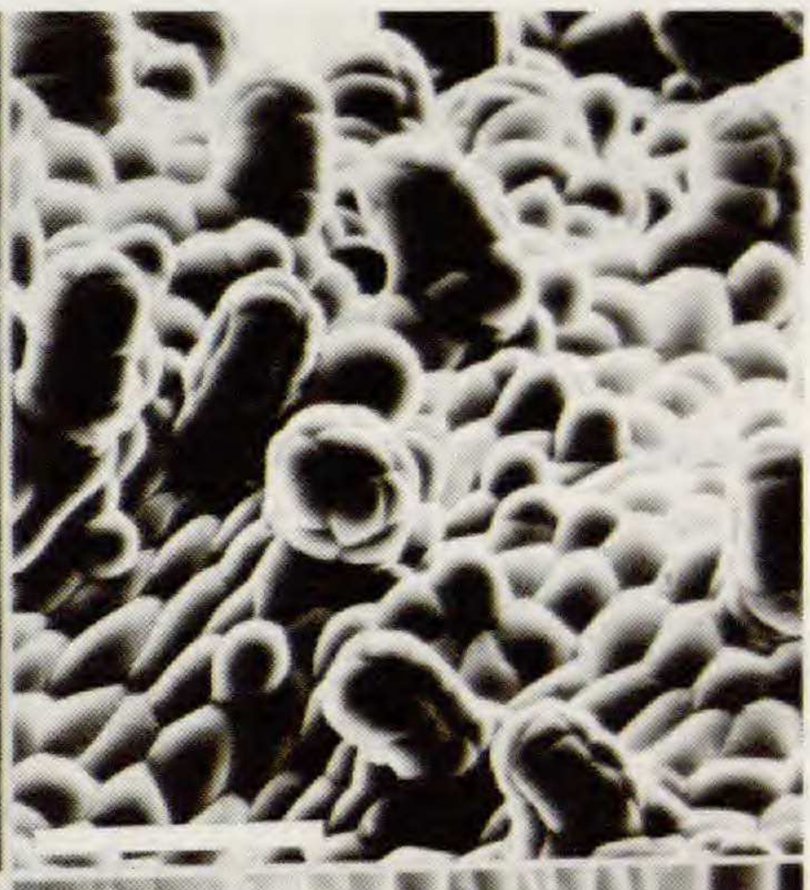


FIGS. 1A–F. All scanning electron micrographs of casts of gametophytes. FIG. 1A. Whole gametophyte of *Pteridium aquilinum* used to give the extract used in the lane on the far right of Fig. 2E. Bar = 1mm. FIG. 1B. Higher magnification of 1A to shqw apical notch and antheridia. Bar = 300μm. FIG. 1C. As in 1B. Note collapsed (senescent) antheridium with superficial fungal hyphae (arrow), and bacterial cells. Bar = 50μm. FIG. 1D. Immature antheridium of *Osmunda regalis*. Note smooth cell to cell boundaries (cf. 1E). Bar = 25μm. FIG. 1E. Mature antheridium from the same gametophyte as 1D. Note pulled-in, puckered cell to cell junctions. Bar = 25μm. FIG. 1F. Trichomes on gametophyte of *Dryopteris oreades*. Bar = 50μm.

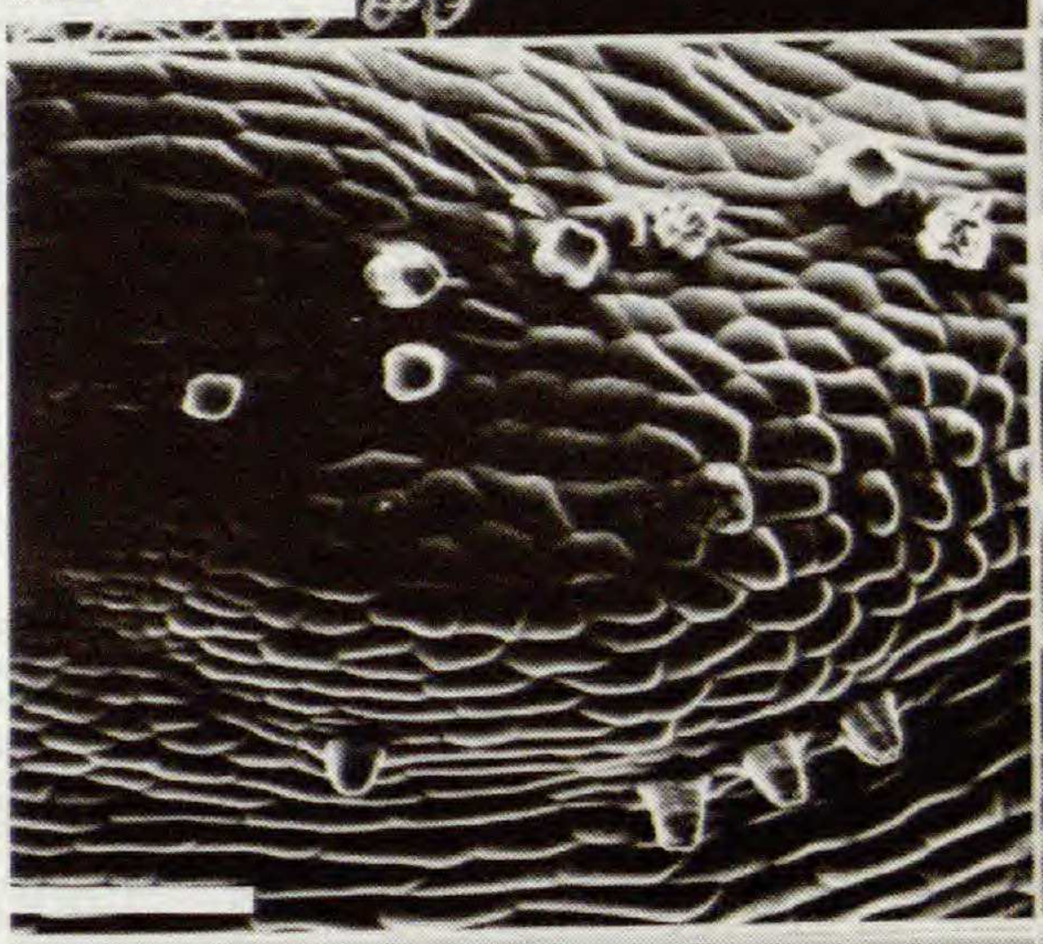
A



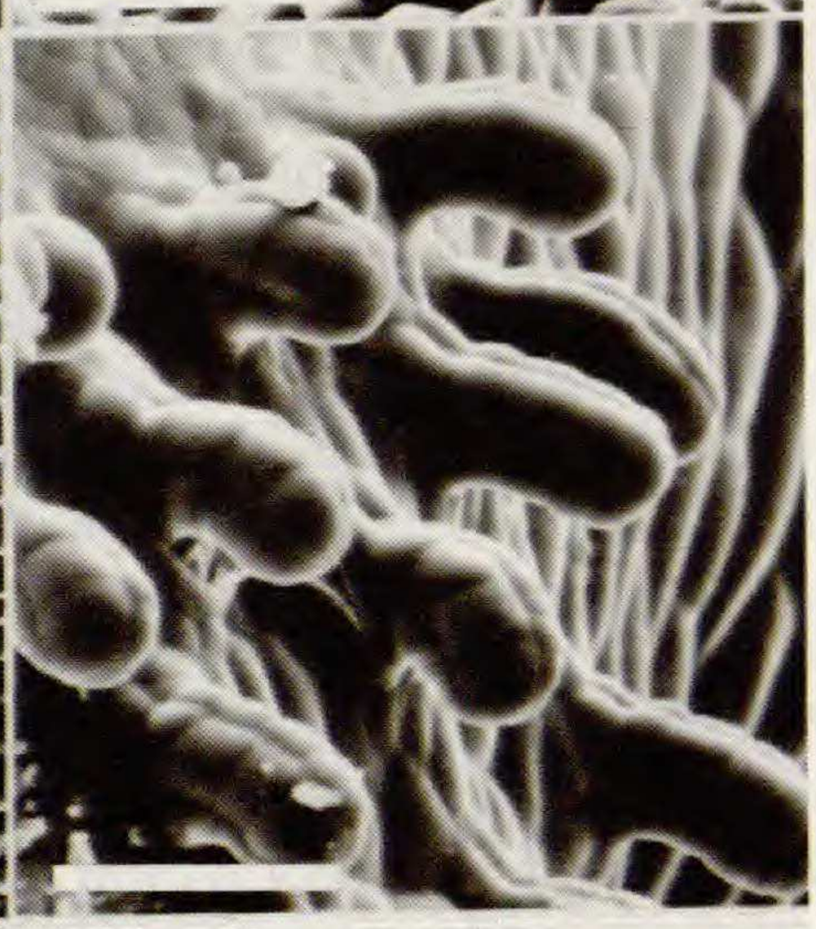
B



C

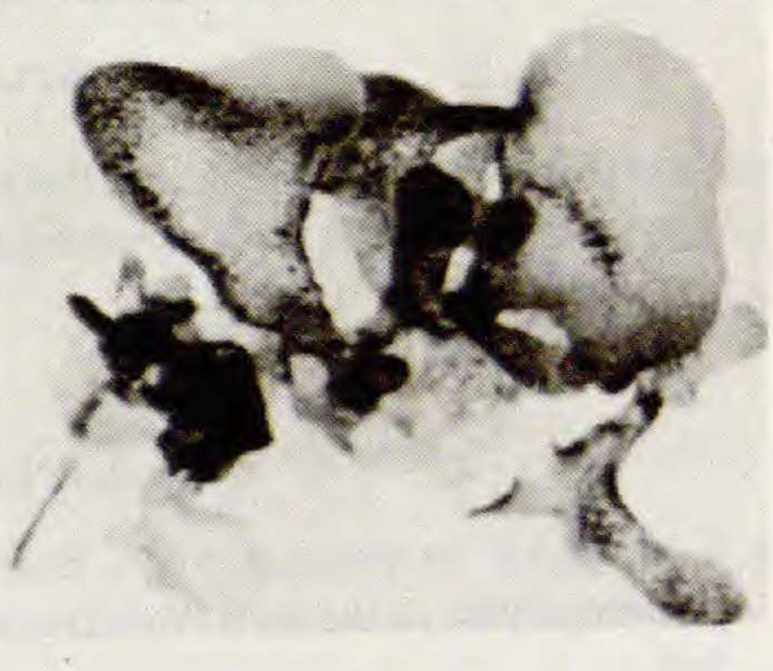


D



E

F



FIGS. 2A–D. All scanning electron micrographs of casts of gametophytes. FIG. 2A. Gametophyte lobe of *Pteridium aquilinum* grown for 8 weeks on a medium containing 5% sucrose. Bar = 400μm. FIG. 2B. Misshapen archegonia and outgrowth of gametophyte as in 2A. Bar = 100μm. FIG. 2C. Gametophyte of *Osmunda regalis* used to give the extract used in the second lane from the right in Fig. 2E, showing archegonia, two of which lack tips, due to air bubbles in the cast-making stage (arrows). Bar = 200μm. FIG. 2D. Archegonia of *Asplenium ceterach*. Bar = 100μm. FIG. 2E. Part of a starch gel stained to reveal activity of the enzyme phosphoglucoisomerase in extracts of single or bisected gametophytes from which impressions had been made. From left to right the species were *Pteris cretica, Dryopteris oreades, Osmunda regalis, Cystopteris dickieana, Asplenium billotii, Anemia phyllitidis, Pteris cretica, Dryopteris oreades, Osmunda regalis, Asplenium billotii, Cysopteris dickieana, Osmunda regalis, Pteridium aquilinum*. FIG. 2F. Tissue regenerated from an *Anemia phyllitidis* gametophyte from which a cast had been made, and which had then been bisected, this half had been returned to culture medium for six weeks, the other half was used to give the extract used in the sixth lane from the left in Fig. 2E.

bisected gametophyte from which an impression and an extract for electrophoresis had been made.

Discussion

Excellent representation of the delicate gametangia and cells of gametophytes was obtained, and artefacts (other than parts missing due to air bubbles) arising from the procedure were not apparent. Gametophytes appeared to be unscathed by the impression-making stage, and their extracts gave clear electrophoretic banding patterns. Regeneration of tissue from homogenized/fragmented gametophytes is well established (e.g. Sheffield & Attree, 1983), and was as successful with gametophytes from which impressions had been made. Low levels of microbial contamination were observed, but did not appear to impede regeneration and could probably have been avoided if the operations had been performed aseptically.

The technique outlined herein therefore provides a quick, cheap, and non-destructive method by which good quality casts of fern gametophytes can be obtained. The casts are good for stereo- or light-microscope study as well as for scanning electron microscopy, and can provide a permanent collection for research and teaching purposes. In addition to the uses outlined here, the gametophytes could be used for breeding, transmission electron microscope or cytological studies (e.g. Wolf, Haufler & Sheffield, 1987), or grown on and impressions made of later developmental stages (cf. Williams & Green, 1988), thus maximizing the information obtainable from a single fern gametophyte.

Acknowledgments

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